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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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IL-31 Induces Chemotaxis, Calcium Mobilization, Release of Reactive Oxygen Species, and CCL26 in Eosinophils, Which Are Capable to Release IL-31

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TO THE EDITOR

Human eosinophils have an important role in the pathogenesis of allergic inflammatory diseases including atopic dermatitis (AD) and allergic diseases (Simon *et al.*, 2004; Rothenberg and Hogan, 2006; Raap and Wardlaw, 2008). Eosinophils secrete proinflammatory cytokines, chemokines, and proteins like eosinophil cationic protein (RNASE3), a protein known to correlate with disease severity in patients with AD (Kapp, 1993). Another cytokine correlating with disease severity in patients with AD is the pruritogenic IL-31 (Raap *et al.*, 2008; Raap *et al.*, 2012). IL-31 was shown to promote chronic dermatitis in mice through the induction of severe itch (Dillon *et al.*, 2004). Findings that a subpopulation of IL-31RA(+)/TRPV1(+)/TRAP1 (+) neurons mediates T-helper cell–dependent itch support the role of IL-31 in pruritus (Cevikbas *et al.*, 2014). In addition, skin IL-31 mRNA expression and IL-31 serum level correlate with Th2 cytokines including IL-4 and IL-13 in AD and acute allergic contact dermatitis (Neis *et al.*, 2006; Raap *et al.*, 2012).

Originally, IL-31 expression was shown in activated $CD4^+$ T-helper cells (Dillon *et al.*, 2004; Cornelissen *et al.*, 2012). We demonstrate the expression of IL-31 in human peripheral blood eosinophils (Figure 1a–h). Freshly isolated eosinophils from non-atopic patients, who gave written informed consent (approved by the ethics committee of the Hannover Medical School (MHH)), were cultivated with and without IL-3, and intracellular IL-31 was measured (Figure 1a, see Supplementary Material and Methods S1 online). Stimulation with IL-3, a cytokine that can enhance responses of eosinophils to various agonists (Simon *et al.*, 2004), increased the intracellular expression of IL-31 (Figure 1a). These results were confirmed with the western blot technique and the densitometric analysis of the western blot (Figure 1b and c). In addition, we determined IL-31 protein content in supernatants by ELISA (Figure 1d). The release of IL-31 increased during the time of incubation of eosinophils (Figure 1d). Similar to the densitometric analysis (Figure 1c) of the western blot, IL-3 significantly increased IL-31

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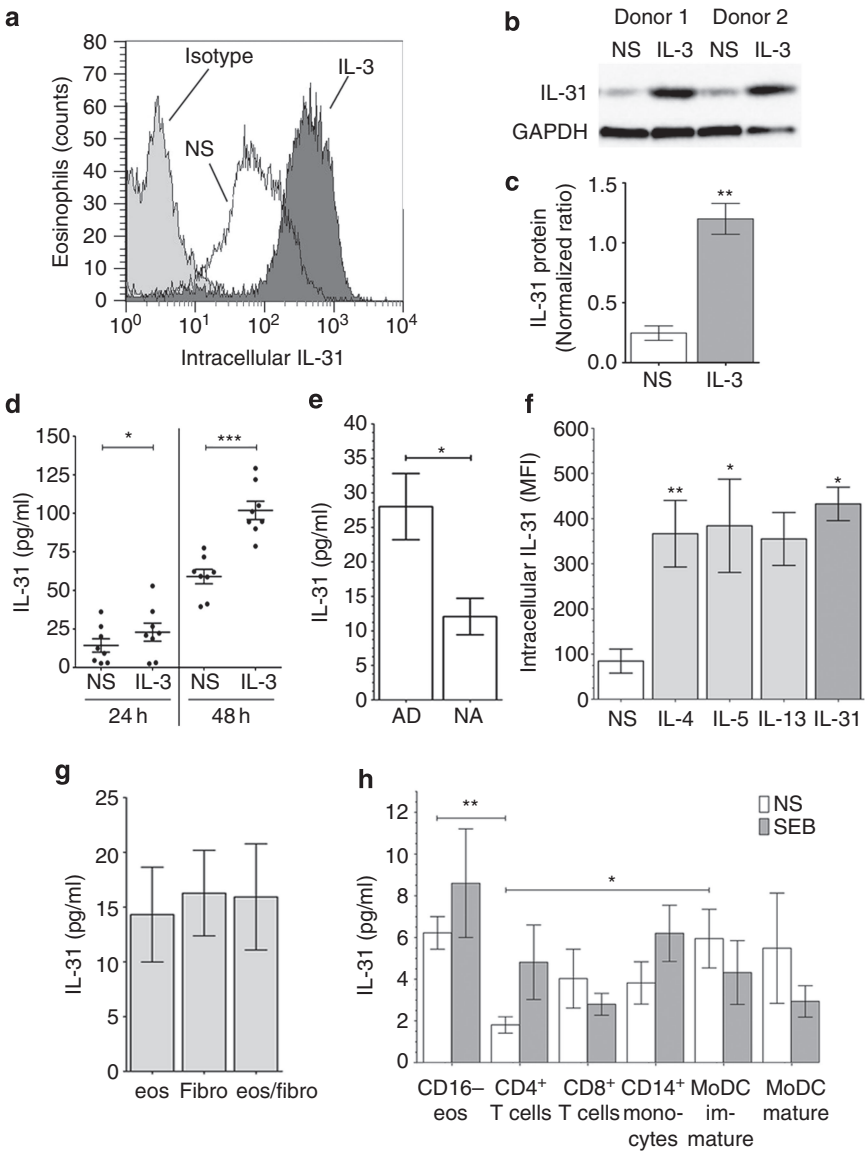

Figure 1. Analysis of the expression and release of IL-31 in eosinophils. Human eosinophils from atopic dermatitis and non-atopic patients were isolated as described in Supplementary Material and Methods (S1 online). Eosinophils were stimulated with various cytokines (each 10 ng ml^{-1}) or SEB (1 μg ml^{-1}) for the respective time (NS: unstimulated) (*$P<0.05$; **$P<0.01$; ***$P<0.001$; SEM). (**a**) Intracellular staining of IL-31 displayed as histogram ($n=10$). After 60 minutes of stimulation, fixed and permeabilized eosinophils (NA) were stained with antibodies against IL-31 or isotype. Light grey area: isotype; colourless area: no stimulation (NS); dark grey area: IL-3 stimulation. (**b**) Detection of IL-31 with western blot (WB) from protein lysate 60 minutes after stimulation (one WB out of three; D1/ D2: Donor; $n=3$, NA). GAPDH was used as a reference gene. (**c**) Densitometric analysis of the WB results by using ImageJ ($n=3$). (**d**) Determination of IL-31 in the supernatant after 24 and 48 hours by ELISA (non-stimulated, $n=8$, NA). (**e**) Determination of IL-31 in the supernatant of eosinophils from atopic dermatitis (non-stimulated, $n=3$, AD) and non-atopic (non-stimulated, $n=3$, NA) patients after 24 hours. (**f**) IL-31 intracellular staining after stimulation with IL-4, IL-13, IL-5, and IL-31 for 60 minutes (MFI: mean of fluorescence intensity; NA). (**g**). Determination of IL-31 in the supernatant of eosinophils (eos, NA), fibroblasts (fibro), and co-culture of both after 24 hours in culture (non-stimulated, $n=3$). (**h**) Determination of IL-31 in the supernatant by ELISA. CD4$^+$ and CD8$^+$ T cells, CD14$^+$ monocytes, eosinophils (CD16$^-$, eos), and monocyte-derived dendritic cells (DC) (MoDC, immature/ mature) were isolated and stimulated with SEB for 6 hours ($n=4$, NA). AD, atopic dermatitis patient; NA, non-atopic patient.

protein release (Figure 1d). Indeed this is interesting given the fact that eosinophils could activate neighboring cells including T cells, mast cells, and epithelial cells in the inflammatory infiltrate via the release of IL-31. Thus, this finding is highly relevant, especially in regard to inflammatory itchy skin diseases and particular as it was shown that IL-31-stimulated T cells produce CCL2 and GM-CSF (Stott *et al.*, 2013).

As IL-31 is increased in allergic inflammatory skin diseases including AD, we determined the release of IL-31 from eosinophils of AD patients, who signed informed consent (approved by the ethics committee of the MHH). AD eosinophils displayed an increased release of IL-31 after 24 hours in culture compared with eosinophils of non-atopic patients (Figure 1e). We thus

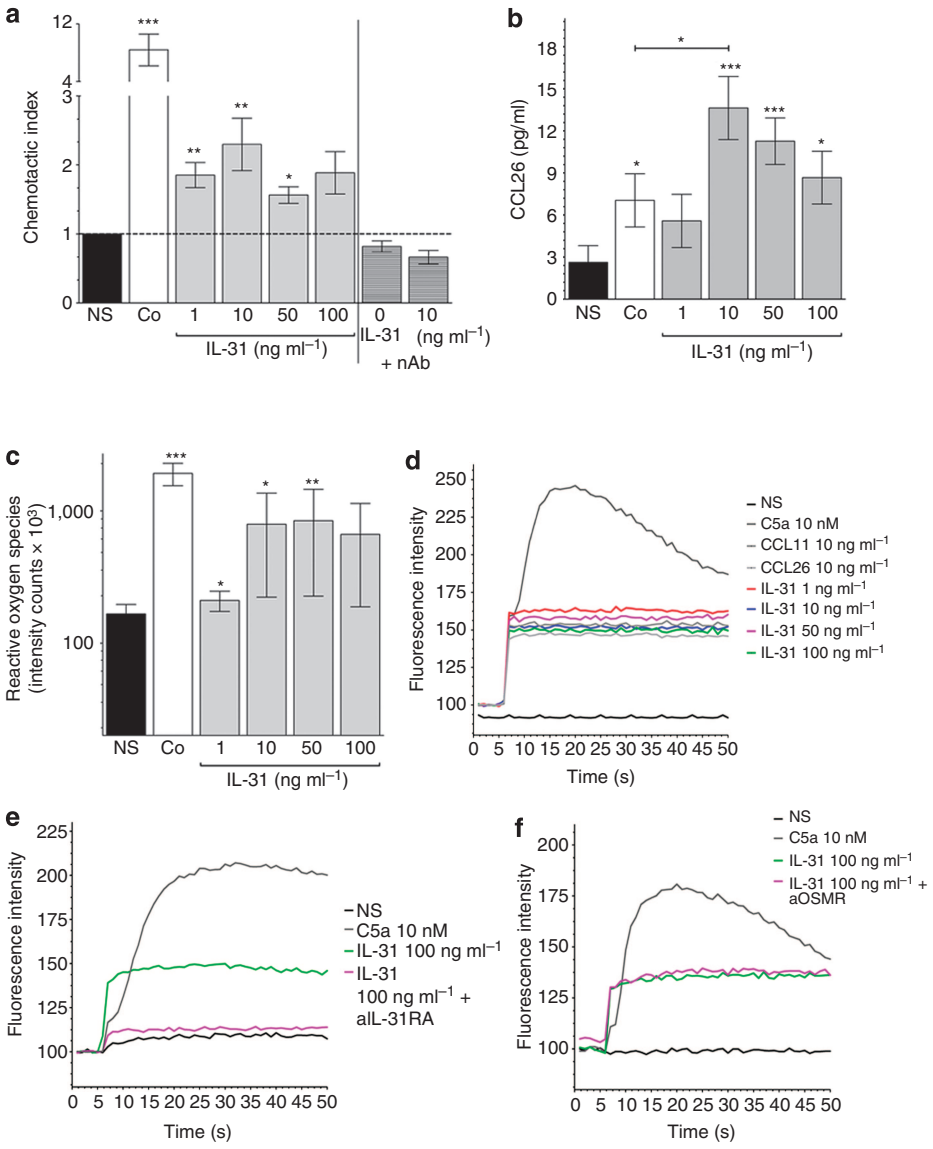

Figure 2. Chemotaxis, ROS, and CCL26 release and calcium mobilization. Human eosinophils from non-atopic patients were isolated as described in Materials and methods and stimulated with IL-31 (1–100 ng ml^{-1}) or C5a as a positive control for the respective time (S1 online). (nAB: 20 μg ml^{-1}; anti-IL31RA Ab 10 μg ml^{-1}; anti-OSMR Ab, 10 μg ml^{-1}), (*P<0.05; **P<0.01; ***P<0.001; SEM). (**a**) Chemotaxis assay: migration of eosinophils during 3 hours was analyzed by using a modified Boyden Chamber in the presence of C5a (Co, 10^{-8} M) and IL-31 (1–100 ng ml^{-1}) (n=15). Neutralizing antibodies against IL-31 were used to block the migration (n=3). Chemotactic activity is presented as the ratio of the number of migrating eosinophils in the presence of stimulus/migrating eosinophils in the presence of the medium. Dotted line indicates the level of chemotaxis of unstimulated (NS) eosinophils. (**b**) Measurement of CCL26 in the supernatants after 48 hours by ELISA after stimulation with IL-3 (positive control, Co, 10 ng ml^{-1}) and IL-31 (n=13). (**c**) Release of ROS (reactive oxygen species) displayed as intensity count (n=20) measurement using a single-photon imaging system followed immediately after stimulation with C5a (Co, 10^{-8} M) or IL-31. (**d**) Calcium influx was measured immediately after stimulation with IL-31 and displayed as fluorescence intensity per seconds (n=3). C5a (10^{-8} M), CCL11, and CCL26 (each 10 ng ml^{-1}) served as positive controls. (**e**) Inhibition of the IL-31 (10 ng ml^{-1}) induced calcium influx by using anti-IL-31RA. (**f**) Measurement of the IL-31 (10 ng ml^{-1}) induced calcium influx after prestimulation with anti-OSMR. Co, pos. control; nAB, neutralizing antibody; NS, non stimulated.

addressed whether Th2 cytokines regulate IL-31 release. Accordingly, eosinophils were cultivated for 60 minutes with IL-4, IL-5, IL-13, and IL-31 with subsequent analysis of intracellular IL-31 (see Supplementary Material and Methods S1 online). Stimulation with Th2 cytokines led to a clear upregulation of IL-31, which was significant with IL-4 and IL-13 (Figure 1f). Interestingly, IL-31 stimulation led to an increased expression of IL-31 as well (Figure 1f).

We further analyzed the impact of a co-culture of eosinophils with fibroblasts in relation to their IL-31 release (generation of fibroblasts see Supplementary Material and Methods S1 online) after 24 hours in culture. As already observed, fibroblasts secrete IL-31 (Cornelissen *et al.*, 2011). However, the co-culture of eosinophils and fibroblasts did not further enhance IL-31 expression (Figure 1g). As we were interested in the expression of IL-31 by eosinophils in comparison with other cells, we isolated CD4$^+$ and CD8$^+$ T cells, CD14$^+$ monocytes, eosinophils

(CD16$^-$ cells), and monocyte-derived dendritic cells (MoDC; immature and mature) and assessed the IL-31 release in supernatants from non-stimulated and staphylococcus enterotoxin B (SEB, 1 μg ml^{-1})-stimulated cells after 6 hours. SEB, an endotoxin from *S.aureus*, is known to rapidly induce IL-31 mRNA expression in atopic individuals (Sonkoly *et al.*, 2006). Interestingly, eosinophils displayed the highest IL-31 release, which was significantly higher compared with the IL-31 release by CD4$^+$ T-helper cells (Figure 1h). Furthermore, SEB led to a clear induction of IL-31 in eosinophils and CD4$^+$ T-helper cells (Figure 1h). As it seems that IL-31 has a chemotactic function (Dillon *et al.*, 2004), we investigated whether IL-31 influences the eosinophil chemotaxis by performing a modified Boyden-chamber assay (Figure 2a). Within the chosen dose range of 1–100 ng ml^{-1}, IL-31 induced significantly the chemotaxis in eosinophils in a dose-dependent manner (Figure 2a). Using a neutralizing anti-IL-31 antibody, the IL-31 induced chemotaxis was completely abolished (Figure 2a). This indicates that IL-31 may be involved in the recruitment of eosinophils, which is consistent with the finding that IL-31 induces cell surface expression of adhesion molecule CD18 (Cheung *et al.*, 2010). As eosinophils represent circulating cells, their recruitment via chemotaxis is important for the maintenance of the inflammatory infiltrate (Rothenberg and Hogan, 2006). In addition, we displayed that IL-31 increased CCL26 release in a dose-dependent manner, which supports the finding that IL-31 induces migration of eosinophils (Figure 2b). In response to a chemoattractant, the concentration of cytoplasmic calcium rises. We tested the calcium influx under the influence of IL-31. In eosinophils from non-atopic patients, the intracellular Ca^{2+} levels increased after stimulation with IL-31 (Figure 2d). As positive controls C5a, CCL11, and CCL26 were used (Lampinen *et al.*, 2004). IL-31 caused an influx of Ca^{2+} similar to CCL11 and CCL26 (Figure 2d). Further, we wanted to see whether the Ca^{2+} mobilization was mediated via these receptors by blocking with anti-IL-31RA and anti-OSMR antibodies. In the presence of antibodies, the IL-31 induced increase in intracellular Ca^{2+} level was reduced. This reduction was more effective by blocking IL-31RA then by blocking the OSM receptor (Figure 2e and f). Thus, our data indicate a biological function of IL-31 exerted via IL-31RA in eosinophils.

Eosinophils are suggested of having a role in tissue damage by the release of reactive oxygen species (Neves and Weller, 2009). Therefore, we analyzed whether IL-31 influences the release of reactive oxygen species of eosinophils. Stimulation with IL-31 in a dosage of 10, 50, and 100 ng ml^{-1} induced a respiratory burst of eosinophils compared with the incubation in a medium (Figure 2c). C5a was used as a positive control and led to a release of reactive oxygen species as expected (Figure 2c).

Summarizing, to our knowledge previously unreported, we show that eosinophils are a source of IL-31 and capable of releasing it. The findings that the IL-31 release by eosinophils is higher in AD patients and Th2 cytokines further increase the IL-31 release in eosinophils suggest an important regulatory pathway for inflammatory skin diseases including AD. Finally, we show that IL-31 affects eosinophils functionally with induction of chemotaxis, mobilization of Ca^{2+}, release of CCL26, and reactive oxygen species. Thus, IL-31 constitutes an attractive target for the treatment in eosinophil-associated allergic inflammatory diseases.

CONFLICT OF INTEREST

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